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Developmentally regulated usage of *Physarum* DNA replication origins

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To determine the extent to which eukaryotic replication origins are developmentally regulated in transcriptionally competent cells, we compared origin use in untreated growing amoebae and plasmodia of *Physarum polycephalum*. At loci that contain genes transcribed in both developmental stages, such as the ribosomal RNA genes and two unlinked actin genes, we show that there is a similar replicational organization, with the same origins used with comparable efficiencies, as shown by two-dimensional agarose-gel electrophoresis. By contrast, we found cell-type-specific replication patterns for the homologous, unlinked *profilin A* (*proA*) and *profilin P* (*proP*) genes. *proA* is replicated from a promoter-proximal origin in amoebae, in which it is highly expressed, and is replicated passively in the plasmodium, in which it is not expressed. Conversely, *proP* is replicated passively and is not expressed in amoebae, but coincides with an efficient origin when highly expressed in the plasmodium. Our results show a reprogramming of S phase that is linked to the reprogramming of transcription during *Physarum* cell differentiation. This is achieved by the use of two classes of promoter-associated replication origins: those that are constitutively active and those that are developmentally regulated. This suggests that replication origins, like genes, are under epigenetic control associated with cellular differentiation.

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INTRODUCTION

The expression of cell-type-specific genes is a major determinant of eukaryotic cellular differentiation. The preferential early replication of transcribed genes in S phase (Simon & Cedar, 1996; Schübeler *et al*., 2002), suggests that a change in the temporal order of genome replication takes place during cellular differentiation. This could be achieved either by modulating the firing order of the replicons or by selecting new origins. So far, no cell-type-specific eukaryotic origins of DNA replication have been identified. The same origins are used in mitotic and premeiotic S phases in *Saccharomyces cerevisiae* (Collins & Newlon, 1994). In *Xenopus laevis* and *Drosophila melanogaster*, most of the initiation sites used during early embryonic development are suppressed concomitantly with the resumption of transcription (Blumenthal *et al*., 1974; Hyrien *et al*., 1995; Sasaki *et al*., 1999), and it is not known whether the same origins are then selected in the different cell lineages of these organisms. In the fly *Sciara coprophila*, however, the same origin seems to be used during the replication and amplification of the DNA puff II/9A genes (Lunyak *et al*., 2002). Similarly, the constitutively expressed lamin B2 gene is replicated from the same site in several human cell lines (Giacca *et al*., 1994; Kumar *et al*., 1996). Even the developmentally regulated human β-globin gene seems to be replicated from a unique origin, firing either early or late, in cell lines that do or do not express the gene (Dhar *et al*., 1988; Kitsberg *et al*., 1993). These observations suggest a reprogramming of S phase during cellular differentiation by a change in the firing order of a fixed set of origins (Gilbert, 2001; Méchali, 2001). By contrast, the silent *Igh* locus, which is traversed by a single fork in murine non-B cells, is replicated earlier, by forks running in both directions, when activated in B cells (Ermakova *et al*., 1999; Zhou *et al*., 2002), suggesting the activation of a putative cell-type-specific origin at this complex locus.

To determine the extent to which eukaryotic origins are developmentally regulated, we compared origin use in two cell types of the slime mould *Physarum polycephalum*. The *Physarum* genome, which is ten times smaller than the mouse or human genomes, encodes two alternating cell types. In the plasmodium, the naturally occurring synchrony of the cell cycle enabled us to precisely map chromosomal origins linked to abundantly transcribed genes by neutral two-dimensional gel electrophoresis (Brewer & Fangman, 1987; Bénard *et al*., 1995, 1996; Bénard & Pierron, 1992). These large plasmodia generate haploid uninucleated amoebae by meiosis and sporulation, and these in turn, through mating, develop into diploid plasmodia.

RESULTS

We first examined the replication of the ribosomal RNA genes, which are present at about 200 copies per haploid genome on extrachromosomal, linear, 60-kb palindromic DNA molecules. In the plasmodium, these molecules are replicated throughout interphase, mainly from one of four potential site-specific origins (Bénard *et al*., 1995). This feature, coupled with the palindromic structure of the molecules, generates a complicated pattern of replication that is similar in untreated amoebae and plasmodia (Fig. 1). Hence, analysis of a fragment upstream of the transcription initiation

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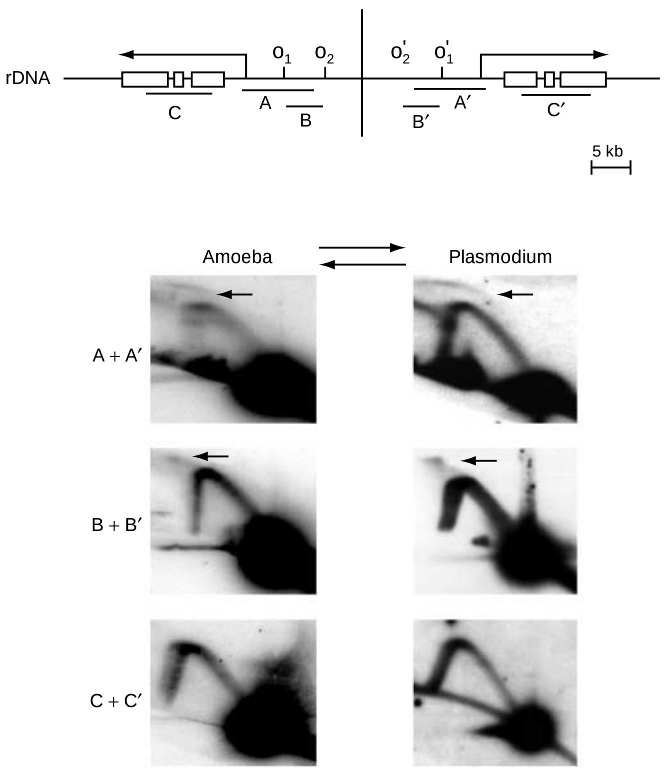

Fig. 1 | Ribosomal DNA replication in two proliferative stages of *Physarum polycephalum*. A map of a palindromic ribosomal DNA molecule is shown (top panel). Arrows indicate the transcriptional polarity of the ribosomal RNA genes. Plasmodial replication origins o_1 and o_2 and their mirror counterparts o_1' and o_2' are shown within the central non-transcribed spacer. The restriction fragments A (7 kb; *Pst*I–*Bam*HI), B (6.4 kb; *Hinc*II) and C (5 kb; *Hind*III) were analysed by two-dimensional gel electrophoresis using total DNA from untreated amoebae and untreated plasmodia. A bubble arc is only observed in the A (and A′) fragment in both cell types. It is associated with a strong Y arc, because A can be passively replicated from initiation in A′, and vice versa. Furthermore, both fragments are replicated passively when initiation takes place at o_2 and o_2'. Electron microscopy analysis has previously revealed that o_2 is approximately twice as active as o_1 (Vogt & Braun, 1977). Taking these facts into consideration, a 1:9 bubble-arc to Y-arc ratio was expected in the A + A′ pattern (Bénard *et al*., 1995). Here, an approximately 1:10 ratio is defined experimentally for both amoebae and plasmodia. Analysis of the B (and B′) inter-origin fragment reveals a strong Y arc and a weak termination signal with amoebal and plasmodial DNAs, indicating a low frequency of simultaneous firing of adjacent origins in both cell types. The intragenic C (and C′) fragment is replicated passively in both cell types, as shown by an arc of simple Ys. Our results show invariant origin usage in the rRNA genes of *Physarum*.

site (fragment A) reveals a composite pattern consisting of a weak bubble-arc and a strong Y arc in both cell types. Phosphorimaging quantitation indicates a bubble-arc to Y-arc ratio of approximately 1:10 in both cell types, indicating similar use of the promoter-proximal origin in amoebae and plasmodia. This conclusion is supported by the identical patterns, consisting of a strong Y arc and a faint double Y arc, that were obtained by probing fragment B. Thus, the simultaneous firing of adjacent origins, although it occurs infrequently, is detectable in both cell types. Finally, when an intragenic fragment (fragment C) is probed, pure Y arcs are generated, indicating a strictly passive replication of the genes. These results show a similar replicational organization of the rRNA genes in proliferative amoebae and plasmodia of *Physarum*.

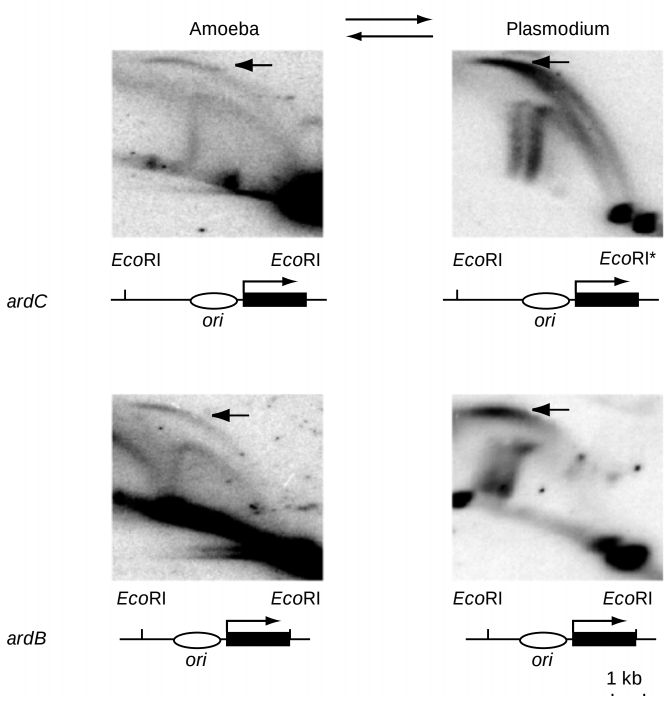

Fig. 2 | Origin use at two unlinked actin loci of *Physarum polycephalum*. Synchronous early S phase plasmodial DNA and total nuclear DNA from asynchronous amoebae was purified. Replication patterns of *Eco*RI fragments encompassing the *ardC* and *ardB* promoters (see maps below micrographs) were determined using specific probes (Bénard *et al*., 1996). For the *ardB* locus, amoebal replication intermediates were purified further by BND (benzoylated naphthaylated DEAE)–cellulose chromatography. Prominent, fully extended bubble arcs show that origins are active at both loci in both cell types (red arrows). The similarity of the two-dimensional gel patterns shows that the same chromosomal origins are used to replicate these two abundantly transcribed actin genes during the amoebal and plasmodial cell cycles (see maps below micrographs). In the diploid plasmodium, because of a polymorphism in the downstream restriction sites (*), the allelic replication intermediates are separated, giving rise to two bubble arcs and two Y arcs (Bénard *et al*., 1996).

The use of a particular replication initiation site, however, cannot be measured easily within a set of repeat sequences. We therefore analysed the activity of single chromosomal origins that are located within the promoter region of two unlinked actin genes, *ardB* and *ardC*. We show that fully extended prominent bubble arcs are detected at both loci in amoebal and plasmodial DNA (Fig. 2), demonstrating that these promoter-associated origins are active in both cell types. For the amoebal DNA, however, the two-dimensional gel patterns are composite, with a complete Y arc underneath the bubble arc. This suggests that replication of the genes from more than one origin occurs in amoebae. However, from the 1:1 bubble-arc to Y-arc ratio on these blots, it is apparent that these promoter-associated origins are not embedded in a cluster of alternately firing origins (compare to the 1:10

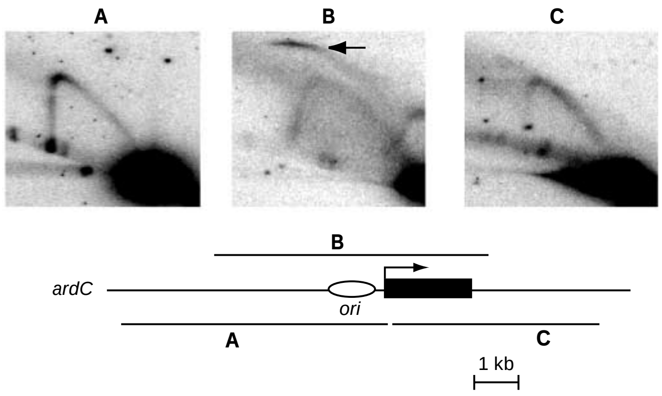

Fig. 3 | The origin of the *ardC* gene is site-specific in amoebae. Overlapping restriction fragments of the *ardC* locus were analysed by two-dimensional gel electrophoresis. Total amoebal DNA (*Eco*RI fragment B) or BND-enriched amoebal DNAs (*Hind*III fragments A and C) were used. Bubble structures (arrow) are only seen in the B fragment, restricting initiations to the centre of this fragment in the amoebae, as previously shown in the plasmodium (Bénard *et al*., 1996). BND, benzoylated naphthaylated DEAE.

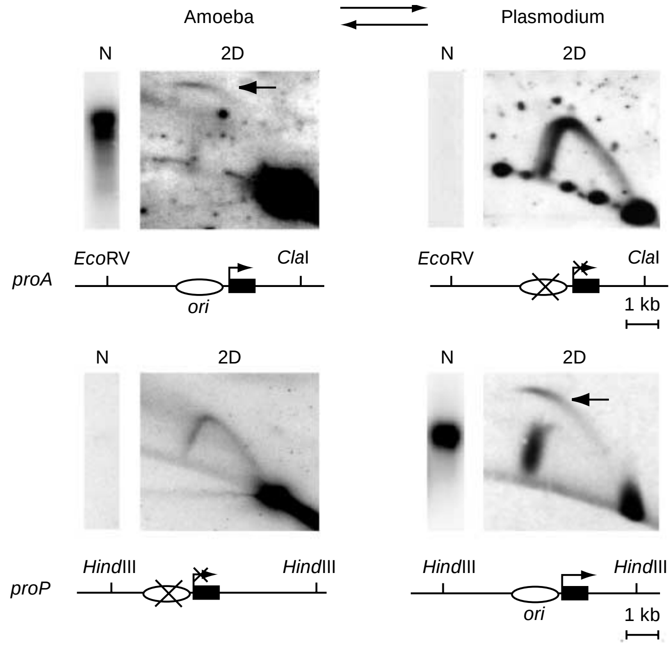

Fig. 4 | Cell-type-specific DNA replication origins in *Physarum polycephalum*. The replication and transcription of two profilin genes were analysed in growing amoebae and plasmodia. Northern blot experiments were carried out using 10 µg of total RNA from each developmental stage to measure messenger RNA levels. Overnight PhosphorImager exposures are shown (N). *proA* gives rise to two abundant mRNAs of 600 nucleotides and 500 nucleotides in amoebae (upper left panel), which are absent from plasmodia (upper right panel; Binette *et al*., 1990). Quantitation indicates that *proA* mRNAs are at least 1,000 times more abundant in amoebae than in plasmodia. A similar bias in favour of *proP* expression in the plasmodium was seen (lower panels; Binette *et al*., 1990). Replication (2D) was analysed using total amoebal DNA for *proA* and BND-enriched amoebal DNA for *proP*. Early (5 min) and mid (60 min)-S-phase plasmodial DNA samples were used for *proP* and *proA*, respectively. Bubble arcs (see red arrows) indicate that *proA* and *proP* are coupled to origins when transcribed. By contrast, these genes are replicated passively when they are not expressed (see maps below micrographs), showing that developmentally regulated origins exist in *Physarum*. BND, benzoylated naphthaylated DEAE.

bubble-arc to Y-arc ratio induced by the four rDNA origins in Fig. 1). Furthermore, no bubble arcs were seen when overlapping fragments of the *ardC* locus were probed with amoebal DNA (Fig. 3), showing that the *ardC* origin is site-specific in amoebae, as it is in the plasmodium (Bénard *et al*., 1996). We conclude that, at the actin and rDNA loci, the same origins are used efficiently in both cell types, showing, consistent with data obtained from other genomes (Giacca *et al*., 1994; Kumar *et al*., 1996; Dhar *et al*., 1988; Kitsberg *et al*., 1993), that a similar set of origins is activated during S phase in differentiated cells. Importantly, *ardB* and *ardC* are among the most abundantly transcribed genes in both amoebae and plasmodia (Hamelin *et al*., 1988). To determine whether cell-type-specific replicons exist, we then studied loci containing developmentally regulated genes.

In *Physarum*, the ubiquitous actin-binding protein, profilin, is encoded by a pair of homologous genes, *proA* and *proP*, whose expression is mutually exclusive. The *proA* gene is highly expressed in amoebae and is not expressed in plasmodia, whereas the converse is true for *proP* (Binette *et al.*, 1990; Fig. 4). We showed previously that in plasmodia the active *proP* gene is replicated at the onset of S phase (0–5 min) from a promoter-proximal, efficient origin (Bénard & Pierron, 1992; Bénard *et al.*, 2001), whereas the silent *proA* gene is replicated in mid-S-phase (40–60 min; Bénard & Pierron, 1992). Our analysis reveals that *proA* coincides with an efficient origin in amoebae. This is shown in Fig. 4 (upper left panel) in the form of a distinct bubble arc, detected on total DNA extracted from untreated asynchronous amoebae. This pattern indicates the presence of an origin in the middle of the fragment, which also contains the promoter region of the gene. By contrast, in plasmodia, the mid-S-phase replication pattern of *proA* is characterized by a prominent Y arc (Fig. 4, upper right panel), indicating passive replication of the locus. This marked decrease in the use of the *proA* origin is therefore correlated with the activity of the gene. The functional organization of *proP* is the mirror image of that of *proA*. We show that *proP* is passively replicated in amoebae (Fig. 4), as a pure Y arc is obtained even using amoebal replication intermediates purified on a BND (benzoylated naphthaylated DEAE) column (Fig. 4). This contrasts with the efficient origin that, paralleling the marked increase in transcription, is detected within the promoter of *proP* in the plasmodium (Fig. 4). Our results show that the activity of these replication origins is highly variable, depending on the differentiation state of the cell. Therefore, in their native chromosomal context, the *proA* and *proP* origins of *Physarum*, like the genes, are developmentally regulated.

DISCUSSION

In this study, we demonstrate a reprogramming of S phase linked to the reprogramming of transcription during eukaryotic cellular differentiation. Two classes of origins are used by plasmodia and amoebae to differentially replicate the *Physarum* genome. At the rRNA and actin loci (Figs 1–3), we show that the same origins are used in both cell types. Using the terminology used for genes, we suggest calling these origins 'housekeeping origins'. By contrast, we provide evidence for 'developmentally regulated' origins for the profilin loci (Fig. 4).

Replication origins are often found in promoter regions of genes, not only in *Physarum*, but also in *Schizosaccharomyces pombe* and in mammalian cells (Giacca *et al*., 1994; Kumar *et al*., 1996; Gomez & Antequera, 1999; Ladenburger *et al*., 2002, Keller *et al*., 2002). Here, we show that there is coordination of the activities of transcription and replication, suggesting a functional interaction in several abundantly transcribed loci (Figs 1,2,4). It is known, however, that transcription *per se* is not necessary to promote the activation of replication origins that are localized upstream of genes, as has been shown for the *hus5* origin of *S. pombe* and at a modified *ARS1* origin in *S. cerevisiae* (Gomez & Antequera, 1999; Bodmer-Glavas *et al*., 2001). When a yeast replication origin is inactivated by deletions of *cis* elements, it can be reactivated by the tethering of a single transcription factor, even in the absence of transcription. This transcription factor can be specific for RNA polymerase II or RNA polymerase III, and can even be chimeric, such as that containing the acidic domain of the p53 tumour suppressor (Bodmer-Glavas *et al*., 2001; Li *et al*., 1998). These observations suggest that a basic function associated with transcription factors affects replication and transcription, possibly by decondensing the chromatin structure. Interestingly, the variation in origin usage that we detected in this study (Fig. 4) is restricted to origins contained in developmentally regulated promoters. It is therefore conceivable that the binding of regulatory factors to these promoters specifies cell-type-specific origins in *Physarum*.

Alternatively, dormant origins can be activated by preventing them from being traversed by an invading fork, as has been shown for the quiescent *HML* mating-type locus of *S. cerevisiae* (Vujcic *et al*., 1999). As a result, the replication of the locus is delayed, and silent origins are activated, albeit inefficiently, in late S phase. If one considers the *proP* locus of *Physarum*, such a model would predict that the gene replicates later in the plasmodium (when the origin is active) than it does in amoebae (when the origin is inactive). We have shown previously that the *proP* origin is efficient and is activated at the onset of S phase in the plasmodium (Bénard & Pierron, 1992; Bénard *et al*., 2001), two properties which are not consistent with activation by delayed replication.

A comparison of origin regions in *Physarum* has not identified an obvious consensus sequence, and no autonomously replicating sequence assay is available. It is therefore not known whether *Physarum* replication origins are encoded by a *cis*-acting DNA sequence or whether they are defined by epigenetic mechanisms, such as chromatin structure (Gilbert, 2001; Méchali, 2001). Amoebae and plasmodia probably use the same mechanisms of origin recognition and activation, as the rRNA and actin-linked replication origins have similar activities in these two untreated cell types. Therefore, when a cell-type-specific origin becomes functional in its native chromosomal context as a result of cellular differentiation, it is likely that epigenetic mechanisms are involved both in its selection and in the inheritance of the replication pattern.

METHODS

Strains and culture conditions. Plasmodia of strain M3CIV and amoebae of strain LU352 were grown under standard conditions (Bénard *et al*., 1996; Dee *et al*., 1989).

Northern blot analysis. Plasmodial and amoebal RNA was prepared as described previously (Maric *et al*., 2002). 10 μg of total RNA was used in northern blot experiments.

DNA isolation. Plasmodial DNA was extracted as described previously (Bénard *et al*., 2001). Amoebal DNA was purified from a 200-ml liquid culture containing 2×10^9 amoebae. Cells were centrifuged for 5 min at 3,000 r.p.m. at 4 °C, washed in cold PBS containing 50 mM EDTA and centrifuged again. Nuclei were isolated by the same procedure used for plasmodia (Bénard *et al*., 1996), lysed in 50 mM Tris, pH 8.0, 50 mM NaCl, 25 mM EDTA, 1% sarkosyl, and were incubated overnight at 45 °C with 200 μg ml^{-1} proteinase K. CsCl and ethidium bromide were added to final concentrations of 915 mg ml^{-1} and 1 μg ml^{-1}, respectively. After centrifugation at 60,000 r.p.m. for 6 h at 20 °C in an NVT90 rotor, the DNA was collected by side puncture under ultraviolet light and dialysed against 10 mM Tris, pH 8.0, 1 mM EDTA.

Two-dimensional gel electrophoresis. Agarose plugs containing ~10 μg of synchronous plasmodial DNA were digested and loaded as described previously (Bénard *et al*., 1996, 2001). 30 μg of asynchronous amoebal DNA was digested, ethanol precipitated and loaded onto the gel. In some experiments, 150 μg of amoebal DNA was digested, and the replication intermediates were enriched on a BND–cellulose column, as described in Hyrien *et al*., 1995. Electrophoresis was carried out as described by Brewer & Fangman (1987). Transfer, hybridization and PhosphorImager exposure of the blots were carried out as described previously (Bénard *et al*., 2001). The probes used have been described previously (Bénard *et al*., 1995, 1996; Bénard & Pierron, 1992).

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